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Type II NAD(P)H dehydrogenases are targeted to mitochondria and chloroplasts or peroxisomes in *Arabidopsis thaliana*

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Abstract **We found that four type II NAD(P)H dehydrogenases (ND) in Arabidopsis are targeted to two locations in the cell; NDC1 was targeted to mitochondria and chloroplasts, while NDA1, NDA2 and NDB1 were targeted to mitochondria and peroxisomes. Targeting of NDC1 to chloroplasts as well as mitochondria was shown using in vitro and in vivo uptake assays and dual targeting of NDC1 to plastids relies on regions in the mature part of the protein. Accumulation of NDA type dehydrogenases to peroxisomes and mitochondria was confirmed using Western blot analysis on highly purified organelle fractions. Targeting of ND proteins to mitochondria and peroxisomes is achieved by two separate signals, a C-terminal signal for peroxisomes and an N-terminal signal for mitochondria.**

Keywords: Chloroplast; Mitochondria; Peroxisome; Dual targeting; Green fluorescent protein; Alternative NAD(P)H dehydrogenase

1. Introduction

A hallmark of eukaryotic cells is the partitioning of various biochemical pathways out of the cytosolic milieu and into discrete organelles. Although the compartmentalisation of various biochemical functions allows specialisation, it requires that many functions are duplicated and thus many enzymatic activities take place in more than one organelle. In the majority of cases these common functions are performed by different proteins, encoded by distinct genes, that are each targeted to a single location in the cell [1]. However, in other cases it appears that the same function in different organelles is carried out by the same protein that is targeted to two locations, a process called dual targeting. This was first reported for glutathione reductase from pea, which is targeted to both mitochondria and chloroplasts [2]. To date, studies in several plants suggest that more than 30 proteins are dual targeted to mitochondria and chloroplasts [3].

The targeting of proteins is routinely assessed by attaching a reporter, most often green fluorescent protein (GFP), to the protein being studied and the intra-cellular distribution of fluorescence measured [4]. This approach is convenient and sensitive and has been used widely to define dual targeting to mitochondria and chloroplasts [5–8]. However, this approach has some limitations that depend on the nature of the constructs. Firstly, for proteins that may be targeted to two locations using two signals in different parts of the protein sequence, GFP fusion to one part of the protein can mask an adjacent signal – resulting in localisation to only one of its in vivo destinations. Secondly, targeting ability can be affected by the nature of the passenger protein. This occurs even for proteins targeted to a single location [9,10], but it seems to be even more pronounced for dual targeted proteins. In two independent studies examining the role of the mature protein for dual targeted proteins to mitochondria and chloroplasts, both concluded that the passenger or mature protein influenced dual targeting ability [11,12].

Type II NAD(P)H dehydrogenases are typically located on the mitochondrial inner membrane where they can oxidise NAD(P)H and are insensitive to the complex I inhibitor rotenone [13–15]. Seven genes encode putative type II NAD(P)H dehydrogenases in Arabidopsis, three have been defined as external (NDB 1, 2 and 4) and three defined as internal NAD(P)H dehydrogenases (NDA 1 and 2 and NDC1) [13,14]. The remaining gene encoding a putative external NAD(P)H dehydrogenase, NDB3, could not be cloned by a number of groups and thus is either a pseudogene or its expression is very restricted [13,16]. Previous studies using GFP tagging have shown NDA1, NDA2, NDB1, NDB2 and NDC1 to be targeted to mitochondria [14], in vitro mitochondrial uptake assays have shown NDA1, NDA2, NDB1, NDB2, NDB4 and NDC1 to be imported into mitochondria [13], and a number of studies using Western blot analysis of mitochondrial proteins and/or cellular fraction with antibodies raised against peptides from potato NDA1 and NDB1 have all concluded a mitochondrial localisation for these proteins [17–19]. Additionally over two decades of biochemical analysis have shown that the

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Abbreviations: AOX, alternative oxidase; GFP, green fluorescent protein; KAT2, 3-ketoacyl-CoA thiolase; ND, type II alternative NAD(P)H dehydrogenase; RFP, red fluorescent protein; SSU, Rubisco small subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase; TIM17-2, translocase of the inner mitochondrial membrane

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activities associated with these proteins are located in mitochondria [15,20]. Thus it can be concluded that these proteins are located in mitochondria.

However the set of proteins predicted to be located in peroxisomes by the AraPerox database, with medium to high confidence, identifies three of these mitochondrial type II NAD(P)H dehydrogenases [21]. Thus we re-assessed the targeting ability of all six NAD(P)H dehydrogenases with the view that they may also be located in other cellular organelles in addition to mitochondria.

2. Materials and methods

2.1. Sequence analysis and cloning

The full length coding sequences of NDA1, NDA2, NDB1, NDB2, NDB4 and NDC1 were cloned as both N- and C-terminal GFP fusions by Gateway cloning under the control of the 35S CaMV promoter. Additionally the last 10 amino acids of NDA1, NDA2, NDB1 and NDB2 were cloned to the C-terminus of GFP. The alternative oxidase (AOX) targeting signal, the full length targeting sequence of small subunit of 1,5 ribulose bisphosphate carboxylase/oxygenase (SSU Rubisco) and the peroxisomal targeting signal SRL of pumpkin malate synthase, were fused to red fluorescent protein (RFP) and used as mitochondrial, chloroplast and peroxisomal controls, respectively [22–24].

The constructs were used to transform Arabidopsis suspension culture cells, Arabidopsis seedlings (1–2 weeks old) and onion epidermal cells by biolistic transformation as previously outlined [25]. Fluorescence patterns were obtained 24 h after transformation by visualization under an Olympus BX61 fluorescence microscope, with excitation wavelengths of 460–480(GFP) and 535–555(RFP). Emissions were collected for GFP between 495 and 540 and RFP between 570 and 625, and imaged using the Cell^R imaging software. To ensure no cross over in detection of signals AOX-RFP and SSU-GFP were co-transformed to ensure that the filters were detecting the appropriate signal.

2.2. Determination of subcellular targeting ability

N- and C-terminal GFP-tagged proteins were used to transform Arabidopsis cell suspension culture, 1–2 week old Arabidopsis seedlings and onion epidermal cells by biolistic transformation as previously outlined [25]. For each construct to be tested three transformations were carried out, the test construct with a mitochondrial, plastidic and peroxisomal control. In vitro import assays into isolated Arabidopsis mitochondria and pea chloroplasts were carried out as previously outlined [23,25].

2.3. Antibody production and Western blotting

Antibodies were raised in rabbit against NDA1, amino acids 57–236 and the NDB2 specific peptide at amino acids 438–452 (ETDDVSKNNIELKIE). The specificity of the antibodies was tested against recombinant proteins synthesised in a wheat germ translation lysate according to manufacturers instructions (Roche, Sydney), programmed to synthesise NDA1, NDA2 and NDB2 by making linear templates by PCR as per manufactures instructions (Roche, Sydney).

Mitochondria and peroxisomes were purified from 7 day old cell suspension culture using free flow electrophoresis as described by Eubel et al. [26]. Western blot analysis was carried out against 20 μg of mitochondrial and peroxisomal proteins separated by SDS–PAGE as previously outlined [27].

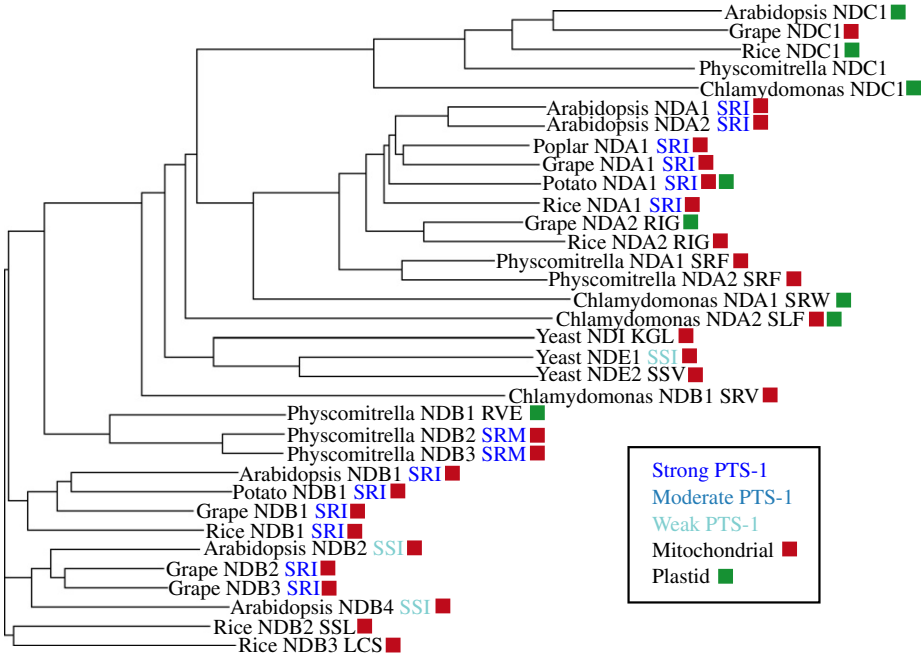

Fig. 1. ClustalW alignment of type II NAD(P)H dehydrogenases from a variety of plants and yeast. Alignment of the sequences encoding type II NAD(P)H dehydrogenases from a variety of plants revealed that several contained putative peroxisomal type I targeting signals (PTSI) at the C-terminal end of the protein. The predicted strength of the PTSI signal was taken from AraPerox [21]. The predicted ability to be targeted to mitochondria and/or chloroplasts is shown. *Arabidopsis thaliana* NDC1 At5g08740;NP_568205, NDA1 At1g07180;NP_563783, NDA2 At2g2990;NP_180560, NDB1 At4g28220;NP_567801, NDB2 At4g05020;NP_180560, NDB4 At2g20800;NP_179673, *Solanum tuberosum* NDA1 CAB52796, NDB1 CAB52797, *Populus trichocarpa* NDA1 ABK95883, *Vitis vinifera* NDA1 CAO21440, NDC1 CAO71655, NDA2 CAO67571, NDB1 CAO41235, NDB2 CAO16606, NDB3 CAO41237, *Saccharomyces cerevisiae* NDI NP_013586, NDE1 NP_013865, NDE2 NP_010198, *Oryza sativa* NDC1 Os06g11140:BAD35311, NDA1 Os01g61410:NP_915326.1, NDA2 Os07g377730:NP911221.1, NDB1 Os06g47000:BAD45556, NDB2 Os05g26660:AAV43826, NDB3 Os08g04630:XP_480031.1, *Chlamydomonas reinhardtii* NDC1 ABR53723, NDA1 XP_001698901, NDA2 XP_001702271, NDB1 XP_001703643, *Physcomitrella patens* NDC1 manually annotated from scaffold 101 of Physcomitrella genome [39], NDA1 manually annotated from scaffold 28 of Physcomitrella genome [39], NDA2 XP_001769969, NDB1 XP_001766162, NDB2 XP_001759207, NDB3 XP_001764062. Targeting prediction for all proteins are shown in Supplementary Table 1.

3. Results

A ClustalW alignment of all ND sequences available from various plant species and yeast revealed the amino acid sequence SRI at the C-terminal end of Arabidopsis NDA1, NDA2 and NDB1 (and NDB3), and in a variety of ND proteins from other plants. Other PTS I type targeting signals, most notably SRM or SSI, were also found in ND sequences [28] (Fig. 1). The fact that these amino acids are not present in all ND sequences suggests that this tripeptide is not required for function, opening the possibility that it may play a role in defining subcellular localisation via its peroxisomal targeting activity [15]. Analysis of NDC1 sequences from Arabidopsis, rice and *Chlamydomonas reinhardtii* predicted plastid-targeting in all three species based on the N-terminal region (Supplementary Table 1), even though these proteins display very low levels of sequence identity in this region (data not shown). Examination of the gene constructs used in a previous study that indicated an exclusive mitochondrial localisation for these proteins, revealed that only the N-terminal region was used in the GFP fusions [14], amino acids 1–55 for NDA1, 1–60 for NDA2, 1–59 for NDB1 and NDB2 and amino acids 1–83 for NDC1.

3.1. NDC1 is targeted to mitochondria and chloroplasts

The full-length cDNA for NDC1 was placed in front of GFP and its subcellular localisation examined by particle bombardment. As controls, the cells transformed with the NDC1-GFP construct were co-transformed either with plastid targeted RFP using the targeting signal of the small subunit of 1,5 ribulose bisphosphate carboxylase/oxygenase (SSU Rubisco-RFP) or the mitochondrial alternative oxidase targeting signal (AOX-RFP). Targeting of NDC1-GFP to chloroplasts was clearly observed in Arabidopsis suspension cells (Fig. 2A), the pattern was clearly not identical to AOX-RFP but resembled that of SSU-RFP quite closely. This is in contrast to what has been previously reported where a mitochondrial localisation was concluded when the first 83 amino acids of NDC1 was used [14]. However we routinely observed a weaker signal, similar to the pattern obtained with AOX-RFP. Thus we tested the targeting ability in a variety of tissues, namely Arabidopsis seedlings and onion epidermal cells. Transformation of these tissues resulted in the detection of two distinct signals, a plastid signal evidenced by relatively large organelles, 2–4 μM in diameter and few in number and smaller organelles, 1 or less μM in diameter typical of a mitochondrial pattern. The mitochondrial targeting ability of NDC1 that we observed in this study is consistent with previous results using GFP and in vitro uptake assays [13,14].

To confirm that NDC1 could target to both chloroplasts and mitochondria, in vitro uptake assays with isolated Arabidopsis mitochondria and pea chloroplasts were carried out. Upon incubation with isolated chloroplasts and mitochondria the NDC1 precursor protein with a mol mass of 70 kDa was imported into a protease resistant location and processed to a mature size with a mol mass of 60 kDa (Fig. 2B, lanes 1–3). Both organelles appeared to process the precursor protein to the same mature protein, to confirm this import reactions into mitochondria and chloroplasts were loaded into the same lane to determine any small difference in mobility, none was

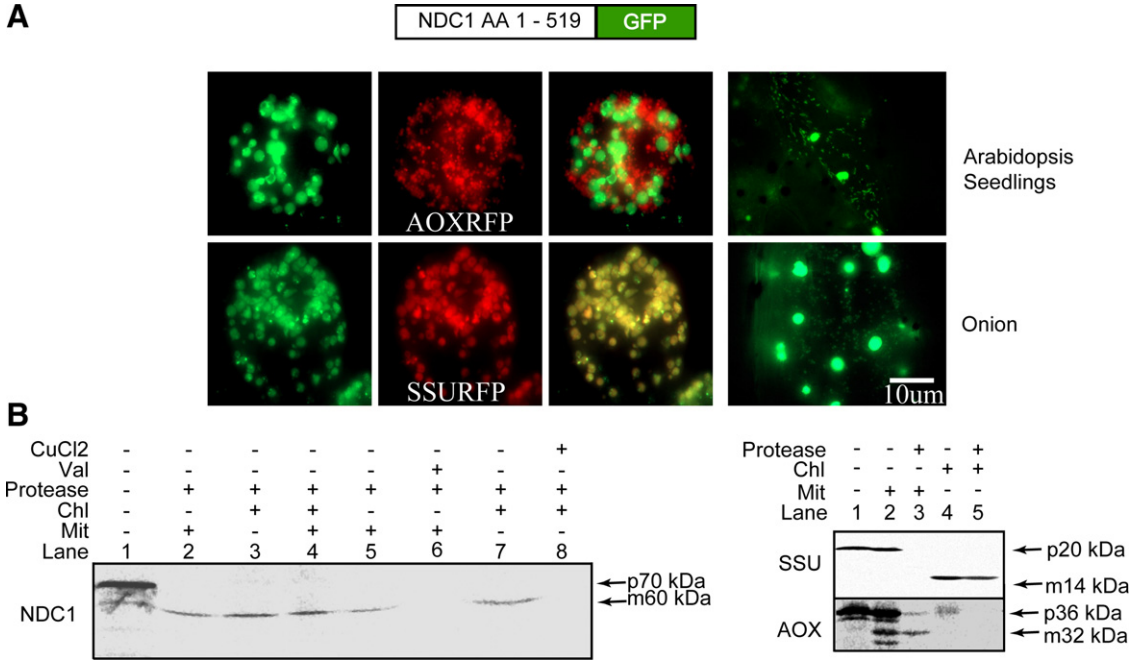

Fig. 2. Subcellular targeting of NDC1 using GFP tagging (A) In vitro uptake assays (B and C). **A**) The full-length cDNA for NDC1 was fused in frame with GFP and co-transformed into Arabidopsis cells with mitochondrial targeted RFP (top panel) or chloroplast targeted RFP (bottom panel). (B) In vitro uptake of NDC1 into isolated mitochondria and chloroplasts. Precursor proteins (lane 1) were incubated with isolated mitochondria (lane 2) and chloroplasts (lane 3) under conditions that support the uptake of proteins into the respective organelles. Uptake was assessed by insensitivity to added protease. Both organelles processed the precursor to a mature protein with the same mobility (lane 4). For mitochondria, uptake was sensitive to the addition of valinomycin (lanes 5 and 6). For chloroplasts uptake was inhibited by addition of $CuCl_2$ (lanes 7 and 8). (C) The specificity of import of protein into isolated organelles was confirmed using the precursor of 1,5 bisphosphate carboxylase oxygenase (SSU) that was only imported into chloroplasts and alternative oxidase (AOX) that was only imported into mitochondria.

detected (Fig. 2B, lane 4). As the translation of the precursor alone also produces a protein with a mol mass of 60 kDa, likely due to translation initiation at an internal methionine, such as amino acid 47 in NDC1. Translation initiation at internal methionine residues is frequently observed with in vitro translation lysates [27]. Thus we confirmed that the protease resistance was due to import into the respective organelle. Import into mitochondria was inhibited by the addition of

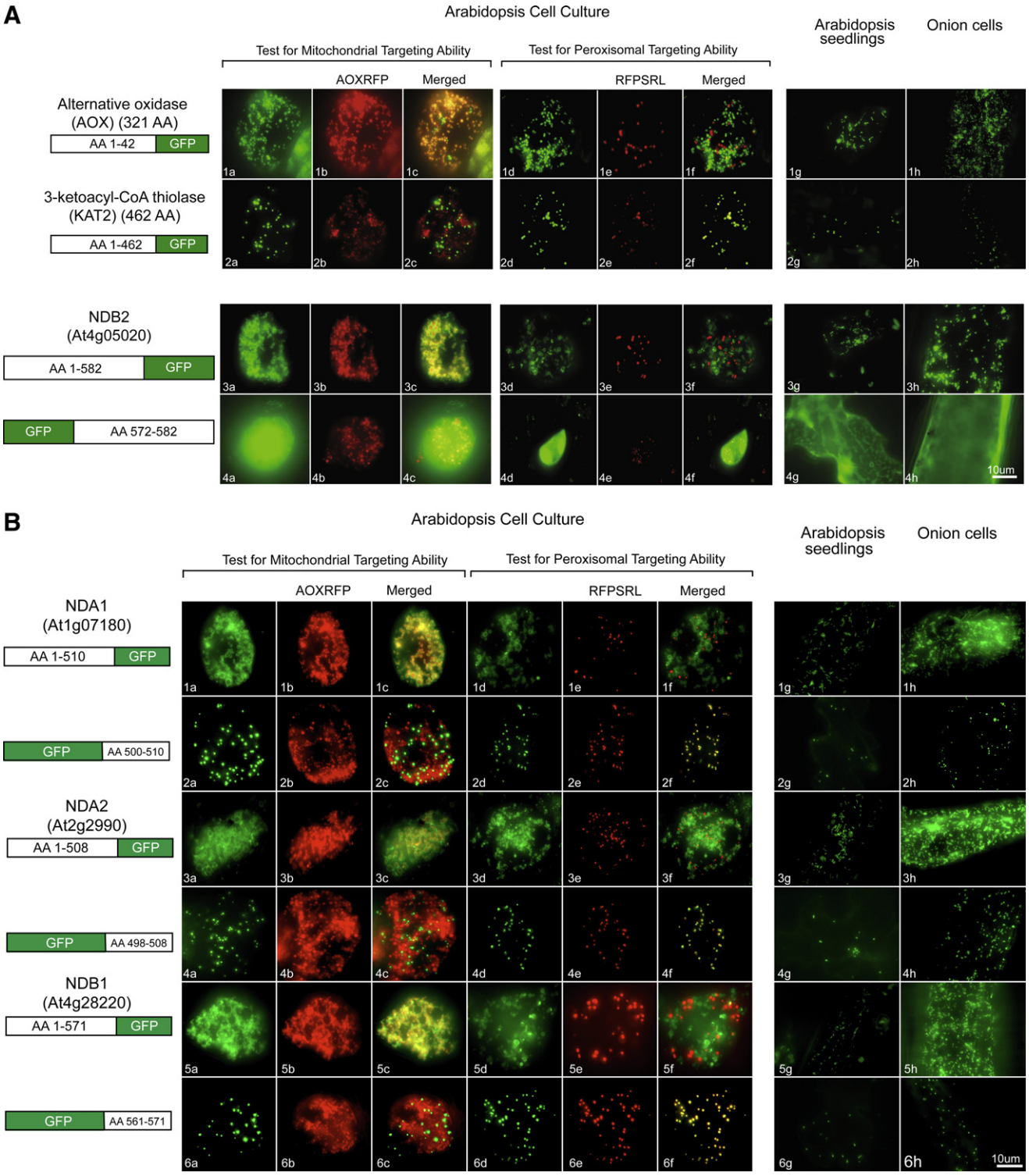

Fig. 3. Subcellular targeting of NDA1, NDA2, NDB1 and NDB2. GFP was fused to the different proteins at the N- or C-terminal and subcellular targeting assessed by particle bombardment of Arabidopsis suspension cells, 1–2 weeks old Arabidopsis seedlings and onion epidermal cells with mitochondrial targeted RFP or peroxisomal targeted RFP as controls. (A) Subcelluar targeting pattern obtained with AOX-GFP, KAT2-GFP and NDB2-GFP. (B) Subcelluar targeting pattern obtained with NDA1, NDA2 and NDB1 fused to GFP. The position of the GFP and the number of amino acids used is shown for each construct.

valinomycin (Fig. 2B, lanes 5 and 6) [27], and import into chloroplast inhibited by the addition of $CuCl_2$ (Fig. 2B, lanes 7 and 8) [29]. The specificity of import into the respective organelles was confirmed as the small subunit of 1,5 bisphosphate (SSU) was only imported into chloroplasts and the alternative oxidase precursor only imported into mitochondria (Fig. 2C, left panel).

3.2. NDA1, NDA2 and NDB1 are targeted to mitochondria and peroxisomes

In order to determine the localisation of the other ND proteins, N- and C-terminal GFP fusions were made followed by particle bombardment. To determine a mitochondrial and peroxisomal pattern chimeric constructs with the AOX and KAT2 linked to GFP were used (Fig. 3A, image series 1 and 2). In the case of NDB2 attaching GFP to the C-terminal resulted in targeting to mitochondria as evidenced by co-localisation with AOX-RFP (Fig. 3A, images 3a–3c). Attaching the last 10 amino acids of NDB2 to the C-terminal end of GFP resulted in a cytosolic localisation for GFP, as evidenced by fluorescence throughout the cell, in all tissues tested (Fig. 3A, image series 4). In contrast when NDA1, NDA2 and NDB1 were tested in a similar manner both mitochondrial and peroxisomal targeting ability was detected. C-terminal fusions gave an exclusively mitochondrial localisation, based on co-localisation with AOX-RFP (Fig. 3B, images 1a–1c, 3a–3c and 5a–5c). This is consistent with the mitochondrial targeting ability previously observed with these proteins [14]. However when the last 10 amino acids of NDA1, NDA2 and NDB1 were placed at the C-terminal region of GFP peroxisomal targeting was observed (Fig. 3B, images 2d–2f, 4d–4f and 6d–6f). The peroxisomal targeting ability of these constructs was also detected in Arabidopsis seedlings and onion cells (Fig. 3B, images 2g and 2h, 4g and 4h and 6g and 6h). Thus we concluded that these proteins were targeted to peroxisomes in addition to mitochondria. NDB4 targeted GFP to mitochondria as previously reported (Supplementary Fig. 1) [14].

To confirm the dual location of NDA1 in mitochondria and peroxisomes we raised antibodies against NDA1, expected to be located in both locations from results above, and NDB2, expected to be located only in mitochondria from results above. We confirmed that the NDA1 and NDB2 antibodies did not cross react with the other antigen by over-expression of the respective proteins in an in vitro translation lysate probing with Anti 6-His antibodies that detected both proteins, Anti A1 antibodies that detected only NDA1 and Anti B2 antibodies that detected only B2 (Fig. 4A). As the NDA1 antibody was raised against a fragment of the NDA1 protein on 180

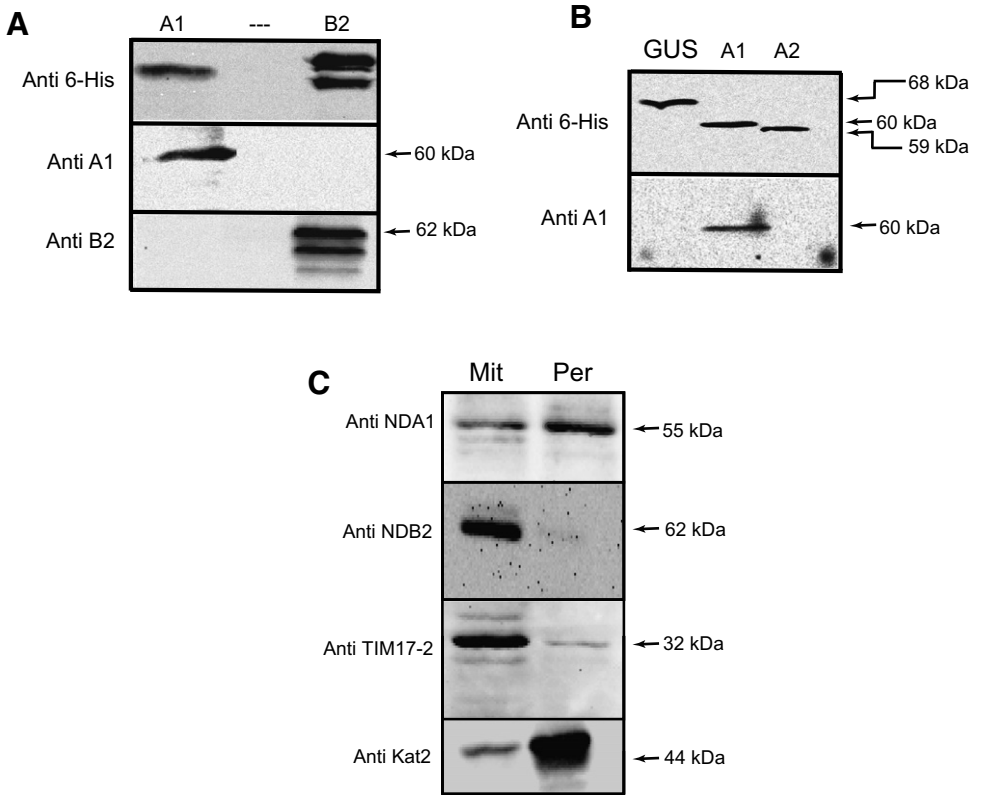

Fig. 4. Western blot analysis of mitochondrial and peroxisomal fractions probed with various antibodies. (A) Confirmation of NDA1 and NDB2 antibodies. Wheat germ lysate (20 μg) programmed to synthesise each of the ND proteins was separated by SDS–PAGE, blotted to a nitrocellulose membrane and probed with antibodies raised against NDA1 and NDB2 to confirm that they detected their target antigens. (B) As A except that translation lysate programmed to synthesis β-glucuronidase (GUS), NDA1 and NDA2. (C) 20 μg of mitochondria or peroxisomes purified by free flow electrophoresis were separated by SDS–PAGE, blotted to a nitrocellulose membrane and probed with antibodies as indicated. The antibody used is indicated to the right of the panel and the apparent mol mass of the cross reacting protein indicated in the left in kDa. Note that for NDA1 and NDA2 the precursor size of the protein is detected when probing in vitro synthesised protein whereas the mature size of the protein is detected when probing organelle fractions.

amino acids that displayed 83% sequence identity with the corresponding region of NDA2 we tested if the NDA1 antibody cross reacted with NDA2. No cross reactivity was detected with full length in vitro synthesised NDA2 (Fig. 4B).

Highly purified mitochondria and peroxisome fractions were isolated from Arabidopsis cells [26] and proteins separated by SDS–PAGE and subjected to Western blotting. In control experiments, we used antibodies against proven markers of mitochondria (TIM17-2 (Translocase of the Inner Mitochondrial membrane; [27]), and peroxisomes (KAT2 (3-ketoacyl-CoA thiolase; [22,30]). These antibodies reacted strongly with mitochondrial and peroxisomal fractions, respectively, and much more weakly with the other fraction, indicating a small degree of cross-contamination between the fractions (Fig.4B). Densitometric analysis revealed the KAT2 signal in mitochondria was ~5% of that detected in peroxisomes, whilst the TIM17-2 signal in peroxisomes was ~1–2% of the signal that could be detected in mitochondria (when the blot was overexposed).

Probing with antibodies raised against NDA1 resulted in the detection of a single protein band with an apparent molecular mass of 55 kDa, in both mitochondrial and peroxisome fractions (Fig. 4B). The blots indicated that there was more NDA1 protein in the peroxisomal fraction than in the mitochondrial one, confirming that these proteins are found in both compartments. Probing mitochondrial and peroxisomal fractions with antibodies raised against the NDB2 specific peptide produced a band only in the mitochondrial fraction (Fig. 4B), confirming that it can target to mitochondria but not to peroxisomes. Importantly, this latter result also shows that the very small amount of cross-contamination between the two isolated fractions cannot explain the dual localisation of the NDA1 signal. Thus the Western blot results confirm the GFP data.

4. Discussion

In this study we have shown that four ND proteins, NDA1, NDA2, NDB1 and NDC1 are dual targeted. The dual targeting ability of ND proteins was overlooked in previous GFP studies due to a number of technical parameters, namely the nature of the GFP-protein constructs used in each study. In the case of NDC1, it appears that the mature protein sequence is required for its dual localisation by GFP (Fig. 2), as observed for other dual targeted proteins [12,31]. The dual targeting of NDA1, NDA2 and NDB1 to mitochondria and peroxisomes is dictated by two distinct signals. In the case of the NDAs, the apparent Mr of the mature protein observed in peroxisomes and mitochondria was identical (Fig. 4C). As NDA proteins are processed upon import into mitochondria [13], this strongly suggests that they are also processed upon import into peroxisomes. It has been shown previously that peroxisomes recognise N-terminal PTS2 type targeting signals that are removed upon import and the processing of the NDA proteins could be carried out by the same peptidase as both NDA1 and NDA2 have a cysteine residue at amino acids 35 and 38, respectively, which defines the processing site by this peptidase [32]. Alternatively, the NDA proteins may be processed by pitrilysin-like metallopeptidase present in peroxisomes [32]. These enzymes belong to the same family of proteases as the mitochondrial processing peptidase [33].

The mitochondrial pattern obtained with GFP with NDA1, NDA2 and NDB1 differed slightly to that obtained from AOX-RFP. Close examination of the merged images revealed that the GFP fluorescence appeared at the periphery of the mitochondrion, thus the GFP and RFP fluorescence co-localise, but are not identical. A similar pattern of GFP fluorescence is routinely obtained when using outer membrane mitochondrial proteins in humans and Arabidopsis [34,35]. This pattern may be due to the fact that GFP attached to the C-terminal of an inner membrane protein will not be 'pulled' into mitochondria. The C-terminal of the ND proteins may be located in the intermembrane space and thus never enter the mitochondrial matrix. Thus the GFP attached to the C-terminal end of these proteins remains outside the mitochondrion. Using only the N-terminal predicted targeting region results in a typical mitochondrial pattern as previously observed [14], as the default targeting information for mitochondria dictated a matrix location [36]. Secondary signals dictate the intra-organelle location and topology of proteins, such as transmembrane regions and the location of positive residues relative to transmembrane regions [36].

The cellular role of various ND proteins now needs to be re-evaluated in light of their dual localisation. For instance NDC1 gene expression is enhanced by light treatments [16] but the protein is also known to be halved in abundance in plastoglobules during high light treatment [37]. So what impact does this transcriptional light response have on the mitochondrial pool of NDC1 protein? Likewise, Western blot analysis with potato mitochondria revealed changes in NDA protein in a diurnal manner [18], and it is now unclear how much of this may be attributed to a mitochondrial function as opposed to a peroxisomal function, or differential contamination of mitochondria with peroxisomes. Further, loss of or over-expression of potato NDB1 has been shown to alter NADPH/NADH ratio in cells [38], but this may be related to its activity in peroxisomes rather than mitochondria.

Seven genes encode alternative ND proteins in Arabidopsis, two NDA like proteins, four NDB type proteins and a single NDC type protein, the latter proposed to be derived from the cyanobacterial ancestor that gave rise to the plastid endosymbiosis [14]. It is tempting to speculate from the prediction of targeting ability of these proteins from a variety of plants (Fig. 1, Supplementary Table 1) that genes encoding single NDA and NDB type proteins underwent duplication followed by acquisition of additional targeting signals by some proteins. In the case of NDC it may have acquired dual targeting ability upon transfer of the gene from the organelle to the nucleus, or alternatively a location specific signal subsequently acquired dual targeting ability over time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.061.

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